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Reshaping a Human Antibody to Inhibit the Interleukin 6-dependent Tumor Cell Growth[1]

Koh Sato, Masayuki Tsuchiya,[2] Jośe Saldanha, Yasuo Koishihara, Yoshiyuki Ohsugi, Tadamitsu Kishimoto, and Mary M. Bendig

Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical, Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412, Japan [K. S., M. T., Y. K., Y. O.]; Medical Research Council Collaborative Center, Mill Hill, London, United Kingdom [K. S., M. T., J. S., M. M. B.]; and Department of Medicine III, Osaka University Medical School, Osaka, Japan [T. K.]

ABSTRACT

The mouse PM-1 monoclonal antibody binds to the human interleukin 6 receptor, inhibits IL-6 functions, and shows strong antitumor cell activity against multiple myeloma cells. In order to be effective as a therapeutic agent administered to human patients in repeated doses, reshaped human PM-1 antibodies consisting of human REI-based light chain and NEW-based heavy chain variable regions were designed and constructed with the assistance of a structural model of the mouse PM-1 variable regions. The best reshaped human PM-1 antibody is equivalent to mouse or chimeric PM-1 antibody in terms of antigen binding and growth inhibition against multiple myeloma cells. Only a few minor changes in the human framework regions were required to recreate the mouse PM-1 antigen-binding site within a human antibody. The reshaped human PM-1 antibody, therefore, could be efficacious in human multiple myeloma patients.

INTRODUCTION

IL-6[3] is a multifunctional cytokine that is produced by a range of cells. It regulates immune responses, acute phase reactions, and hematopoiesis, and may play a central role in host defense mechanisms (1). IL-6R is expressed on lymphoid as well as nonlymphoid cells in accordance with the multifunctional properties of IL-6. Abnormal expression of the IL-6 gene has been suggested to be involved in the pathogenesis of a variety of diseases especially autoimmune disease, mesangial proliferative glomerulonephritis, and plasmacytoma/myeloma (2, 3). Antibodies which inhibit IL-6 functions, therefore, are expected to be useful as therapeutic agents in human patients. Indeed, a clinical study using a mouse monoclonal antibody against human IL-6 to treat a terminally ill patient with primary plasma cell leukemia showed a blockage in myeloma cell proliferation, transient tumor cytostasis, and a reduction in IL-6-related toxicities (4). A mouse monoclonal antibody which binds to the human IL-6R and inhibits IL-6 functions has been isolated (5). This mouse antibody, PM-1, has been demonstrated to have strong antitumor cell activity against human myeloma cells transplanted into the severe combined immunodeficiency mouse (6).

Mouse antibodies, however, are highly immunogenic in human patients. For this reason, their therapeutic value in human patients is limited. In order to be effective as therapeutic agents administered to human patients in repeated doses, mouse antibodies must be engineered to look like human antibodies. The most complete humanization of a mouse antibody is achieved by grafting the CDRs from the mouse antibody into a human antibody to recreate a good, functional antigen-binding site in a reshaped human antibody (7–14).

This report describes the creation of a reshaped human antibody that is equivalent to the original mouse PM-1 antibody in terms of binding to the human IL-6R and inhibition of multiple myeloma cell growth.

MATERIALS AND METHODS

cDNA Cloning. Total RNA was prepared from hybridoma cells using a standard guanidinium thiocyanate/cesium chloride procedure (15). First stranded cDNA synthesis was achieved directly from 5 μg of total RNA (16). cDNAs were generated by a PCR method designed for rapidly cloning entire mouse immunoglobulin variable regions (17).

Construction of Chimeric Antibody. The cDNAs coding for the mouse PM-1 κ light chain or the heavy chain variable regions were modified by a PCR method (10) and then linked to the gene coding for human κ or human γ-1 constant regions, respectively, on the HEF expression vectors.[4] The HEF expression vectors were constructed by replacing human cytomegalovirus promoter/enhancer region of the human cytomegalovirus expresson vectors (9, 10) with human elongation factor 1-α promoter/enhancer region (kindly provided by Dr. Y. Kaziro).

Construction of Reshaped Human Antibodies. Following a PCR mutagenesis method (18), CDRs of the mouse PM-1 light and heavy chain variable regions were grafted to human REI and NEW FRs, respectively. In order to prepare the template DNAs containing the selected human FRs, the *Nco*I-*Bam*HI fragments containing DNA sequences coding for the reshaped human D1.3 light and heavy chain variable regions, which have FRs from human REI and NEW (8), respectively, were excised from plasmid DNAs alys11 and F10 (provided by Dr. G. Winter, MRC, Cambridge, United Kingdom) and then subcloned into the *Hin*dIII-*Bam*HI site of pUC19 vector using a *Hin*dIII-*Nco*I adaptor. Fig. 1 shows the strategy for the construction of the first versions of reshaped human PM-1 light and heavy chain variable regions. Using appropriate mutagenic PCR primers and template DNA, several versions were constructed. After DNA sequencing, the *Hin*dIII-*Bam*HI fragments coding for reshaped human PM-1 variable regions were excised from the pUC19 vectors and inserted in the *Hin*dIII-*Bam*HI sites in the HEF expression vectors.

Transfection of COS Cells and Purification of Antibody. Plasmid DNAs (10 μg of each) were added to a 0.8 ml of 1×10^7 cells/ml in phosphate-buffered saline. A pulse was delivered at 1.9 kV, 25 μF capacitance (Gene Pulsar; Bio-Rad). After a 10-min recovery period at room temperature, the electroporated cells were added to 20 ml of Dulbecco's modified Eagle's medium (GIBCO) containing 10% γ-globulin-free fetal calf serum. After 72 h incubation, the medium was concentrated using a M_r 100,000 cutoff ultrafiltration device (Amicon), and then applied to a protein A-agarose column (Affi-Gel Protein A MAPSII kit; Bio-Rad). The eluate was concentrated and the buffer changed to phosphate-buffered saline using a microconcentrator (Centricon 10; Amicon).

Enzyme-linked Immunosorbent Assay. For the antigen-binding assay, 96-well plates were coated with mouse MT18 monoclonal antibody to the human IL-6R (5), which recognizes an epitope different from that of PM-1 antibody. Following blocking, soluble recombinant human IL-6 receptor (SR344) (19) was added. After a washing, the samples were serially diluted and added to each well and then alkaline phosphatase-conjugated goat anti-human IgG was added. After incubation and washing, substrate buffer was added, and then the absorbance at 405 nm was measured. For the competition assay to determine whether the antibodies could compete with biotinylated IL-6 for binding to IL-6 receptor, 96-well plates were prepared in the same manner as

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[1] This work was a collaborative project between Chugai Pharmaceutical Co., Ltd., and MRC Collaborative Centre.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: IL-6, interleukin 6; cDNA, complementary DNA; CDR, complementarity determining region; FR, framework region; PCR, polymerase chain reaction; HEF, human elongation factor; IL-6R, the specific receptor for IL-6.

[4] Manuscript in preparation.

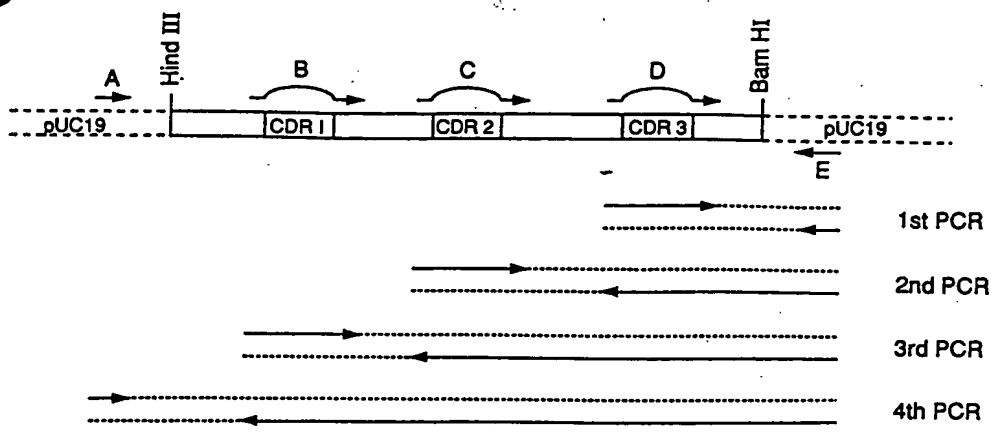


Fig. 1. The CDR-grafting method. Five PCR primers were designed. A backward primer A and a forward primer E hybridized to DNA sequences on the vector. The mutagenic primers B, C, and D were 40–60 bases long, consisting of DNA sequences coding for CDRs from mouse PM-1 variable regions and the FRs in the template DNA that flank the CDR regions. In the first PCR, primers D and E were used. The first PCR product was used in the second PCR as a forward primer with primer C. In the same manner, the third and fourth PCRs were carried out, and then the fourth PCR product was digested with *Bam*HI and *Hin*dII and subcloned into vectors.

described above. The samples were serially diluted and added together with biotinylated human IL-6 to each well, and then alkaline phosphatase-conjugated streptavidin was added. The absorbance at 405 nm was measured as described above.

Cell Lines and Growth Inhibition Assay. Human multiple myeloma cell lines, MMS1 (20) and ILKM3 (21), were maintained in RPMI 1640 supplemented with 10% or 20% fetal calf serum (GIBCO), respectively, in the presence of 250 pg/ml of recombinant human IL-6. S6B45 cell line which was established by transfecting the human IL-6 cDNA into MMS1 cell (22) shows autocrine growth through IL-6 and was maintained in RPMI1640 supplemented with 20% fetal calf serum. For the growth inhibition assay, S6B45 (1×10^4 cells) and ILKM3 (1×10^3 cells), and MMS1 (4×10^4 cells) were cultured in 96-well microplates in the presence of test samples (antibodies) for 4 and 6 days, respectively. After pulse labeling with [^{3}H]thymidine for 5 h, cells were harvested and supplied to a liquid scintillation counter.

Molecular Modeling. A molecular model of the mouse PM-1 antibody variable region was built on a Silicon Graphics Iris 4D work station using the molecular modeling package Quanta (Polygen Corp.). The search of candidate loop structures was made over a database containing 104 high resolution X-ray protein structures using the algorithm of Jones and Thirup (23). The model was energy minimized using the CHARMm potential (24) as implemented in Quanta.

RESULTS

V-Region Sequence Analysis. The mouse PM-1 light and heavy chain variable regions belong to mouse kappa light chain subgroup I and mouse heavy chain subgroup II, respectively. With respect to human antibodies, the mouse PM-1 light and heavy chain variable regions were most similar to REI (72.2%) (25), a member of human κ light chain subgroup I, and VAP (71.8%) (26), a member of human heavy chain subgroup II, respectively. The structures chosen for the modeling of the mouse PM-1 variable regions were those with the best identities, mouse antibodies R19.9 (91.6%) (27) for the light chain and HyHEL-10 (80.6%) (28) for the heavy chain. Canonical structures for the light chain CDRs all corresponded to R19.9; therefore, these were retained in the model. For the heavy chain, only the H2 corresponded to HyHEL-10. The H1 loop was anchored on the α-carbon atoms of residues 29 and 30 and residues 36 and 37. A database search revealed a loop from human ubiquitin (29) from residue 61 (sequential numbering) onwards. Similarly, the H3 loop was anchored on residues 93 and 94, and residues 103 and 104, respectively. A candidate loop from *Pseudomonas* cytochrome *c* (30) was found in the region of residue 55 onwards. The structure model of the mouse PM1 variable regions is shown in Fig. 2.

Design of Reshaped Human PM-1 Variable Regions. As shown in Fig. 3, two versions of reshaped human PM-1 light chain variable region were designed. In version a, the human FRs were identical to the REI-based FRs present in the reshaped human CAMPATH-1H (7). REI is a member of subgroup I of human κ light chain variable regions. Mouse PM-1 kappa light chain variable region is most similar to this human subgroup. Version b was based on version a with only one amino acid change at position 71 in human FR3. Residue 71 is part of the canonical structure for L1 and may, therefore, influence antigen binding. For the reshaped human PM-1 heavy chain variable region, six versions were designed. In all six versions, the human FRs were based on the NEW FRs present in reshaped human CAMPATH-1H. NEW is a member of subgroup II of human heavy chain variable regions and shows 68.4% similarity to mouse PM-1 heavy chain variable region . Seven amino acid residues in the human FRs (positions 1, 27, 28, 29, 30, 48, and 71) were identified as having a possible adverse influence on antigen binding. In the model of mouse PM-1 variable regions, residue 1 is a surface residue that is located close to the CDRs. Residues 27 to 30 are part of the canonical structure for H1 (31) and are observed in the PM-1 model to form part of the H1 loop. These residues, therefore, are likely to be directly involved in antigen binding. Residue 48 is buried under the H2 loop and, therefore, may be influencing the conformation of this loop. Residue 71 is part of the canonical structure for H2 (31–33). In the model, it appeared that Arg-71 influences both the H1 and H2 loop conformations by forming hydrogen bonds with Thr-30, Asp-32, and Ser-54.

Analysis of Reshaped Human PM-1 Antibodies. Two versions of the reshaped human PM-1 light chain were evaluated by combination with the chimeric PM-1 heavy chain. The antigen-binding assay showed that the light chain version a was equivalent to chimeric PM-1 light chain, whereas version b virtually abolished binding to antigen (data not shown). From these results, it was concluded that the change at position 71 in FR3 from a phenylalanine to a tyrosine was very important to recreate a functional antigen-binding site.

Six versions of the reshaped human PM-1 heavy chain were evaluated by combination with the light chain version a (Fig. 4*A*). The light chain version a plus the heavy chain version f clearly provided the best reshaped human PM-1 antibody. It binds to the human IL-6R as well as chimeric PM-1 antibody. It also inhibits human IL-6 from binding to the human IL-6 receptor as well as both original mouse and chimeric PM-1 antibodies (Fig. 4*B*). Versions b, c, and e showed good antigen binding but are not equivalent to chimeric PM-1 antibody (Fig. 4*A*). Version d showed fair binding and version a showed poor binding to the human IL-6 receptor (Fig. 4*A*).

Growth Inhibition against Multiple Myeloma Cells. Fig. 5 showed that the reshaped human PM-1 antibody (a combination of the light chain version a and the heavy chain version f) is effective in inhibiting the IL-6-dependent multiple myeloma cell growth. The concentrations of the reshaped human PM-1 antibody which inhibited 50% cell growth were 90 ng/ml (S6B45), 100 ng/ml (ILKM3), and 0.8

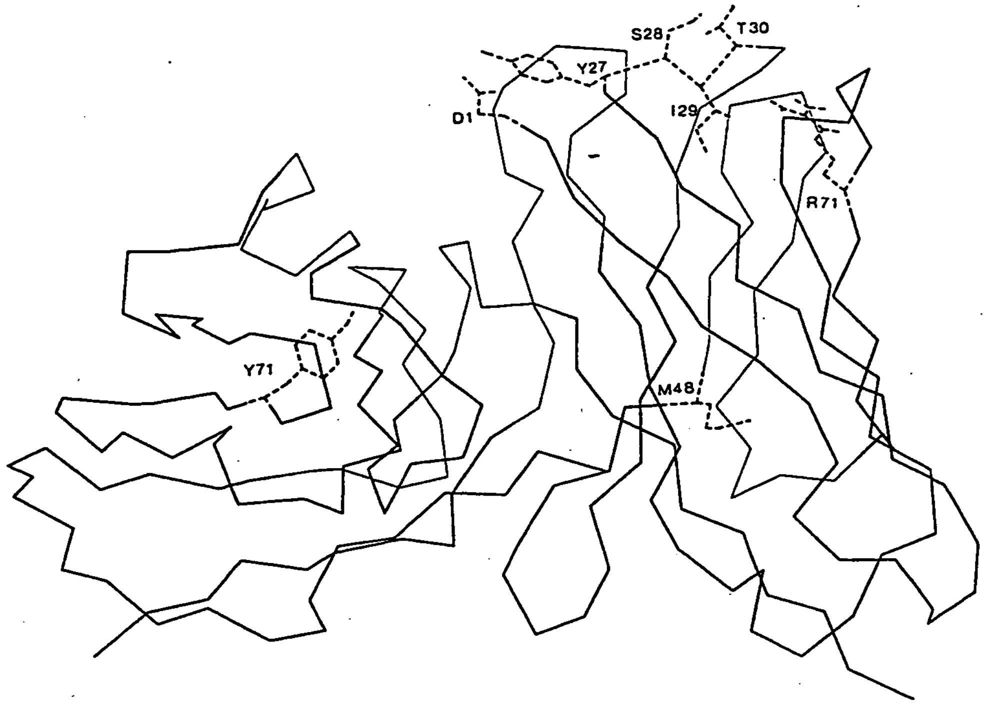


Fig. 2. An α-carbon trace of the variable regions of the mouse PM-1 monoclonal antibody model. The CDRs are shown by *faint lines*, FRs 3 by *bold lines*, and framework side chains of interest in this work by *dotted lines*.

(A)

```
                  FR1                        CDR1
                   1         2                  3
          12345678901234567890123   45678901234

VLPM-1    DIQMTQTTSSLSASLGDRVTISC   RASQDISSYLN
REI       DIQMTQSPSSLSASVGDRVTITC
RVLa      DIQMTQSPSSLSASVGDRVTITC   RASQDISSYLN
RVLb      -----------------------   -----------

              FR2         CDR2
               4          5
          567890123456789   0123456

VLPM-1    WYQQKPDGTIKLLIY   YTSRLHS
REI       WYQQKPGKAPKLLIY
RVLa      WYQQKPGKAPKLLIY   YTSRLHS
RVLb      ---------------   -------

                        FR3                         CDR3
             6         7         8                  9
          78901234567890123456789012345678   901234567

VLPM-1    GVPSRFSGSGSGTDYSLTINNLEQEDIATYFC   QQGNTLPYT
REI       GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC
RVLa      GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC   QQGNTLPYT
RVLb      --------------Y-----------------   ---------

            RF4
           1
           0
          8901234567

VLPM-1    FGGGTKLEIN
REI       FGQGTKVEIK
RVLa      FGQGTKVEIK
RVLb      ----------
```

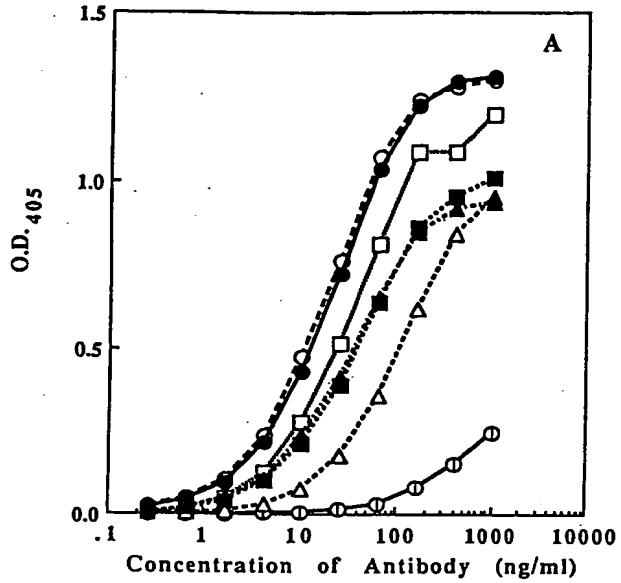
(B)

```
                      FR1                    CDR1        FR2
                   1         2         3                  4
          123456789012345678901234567890    123455    67890123456789
                                                 A
VHPM-1    DVQLQESGPVLVKPSQSLSLTCTVTGYSIT    SDHAWS    WIRQFPGNKLEWMG
NEW       QVQLQESGPGLVRPSQTLSLTCTVSGSTFS              WVRQPPGRGLEWIG
RVHa      QVQLQESGPGLVRPSQTLSLTCTVSGYTFT    SDHAWS    WVRQPPGRGLEWIG
RVHb      --------------------------Y--T    ------    --------------
RVHc      D-------------------------Y--T    ------    --------------
RVHd      --------------------------Y--T    ------    ------------M-
RVHe      D-------------------------Y--T    ------    ------------M-
RVHf      --------------------------YSIT    ------    --------------

              CDR2                          FR3
          5         6          7         8             9
          0123456789012345    67890123456789012222345678901234
                                               ABC
VHPM-1    YISYSGITTYNPSLKS    RISITRDTSKNQFFLQLNSVTTGDTSTYYCAR
NEW                           RVTMLVDTSKNQFSLRLSSVTAADTAVYYCAR
RVHa      YISYSGITTYNPSLKS    RVTMLVDTSKNQFSLRLSSVTAADTAVYYCAR
RVHb      ----------------    -----R--------------------------
RVHc      ----------------    -----R--------------------------
RVHd      ----------------    -----R--------------------------
RVHe      ----------------    -----R--------------------------
RVHf      ----------------    -----R--------------------------

            CDR3         FR4
             1             1
             0             1
          5678900012    34567890123
              AB
VHPM-1    SLARTTAMDY    WGQGTSVTVSS
NEW                     WGQGSLVTVSS
RVHa      SLARTTAMDY    WGQGSLVTVSS
RVHb      ----------    -----------
RVHc      ----------    -----------
RVHd      ----------    -----------
RVHe      ----------    -----------
RVHf      ----------    -----------
```

Fig. 3. Design of different versions of reshaped human PM-1 light and heavy chain variable regions. Amino acid are numbered according to Kabat *et al.* (*A*) The FRs given for REI are those found in the reshaped human CAMPATH-1H light chain (7). The five *underlined* amino acid residues are those that differ from the amino acid sequence of human REI (25). (*B*) The FRs given for NEW are those found in the first version of reshaped human CAMPATH-1H heavy chain.



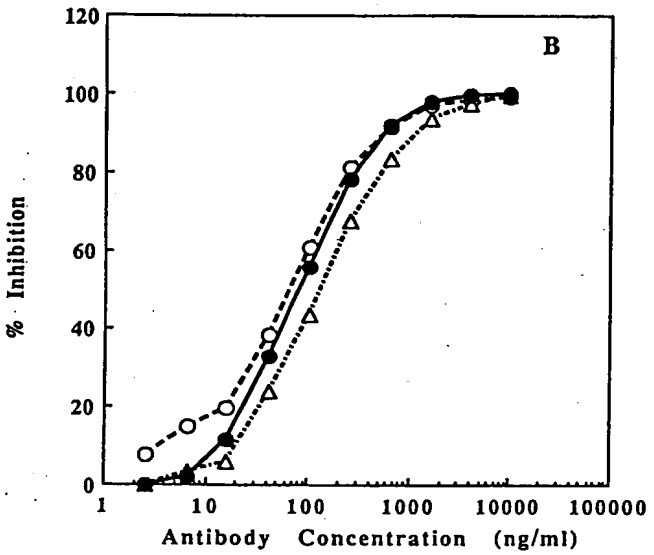
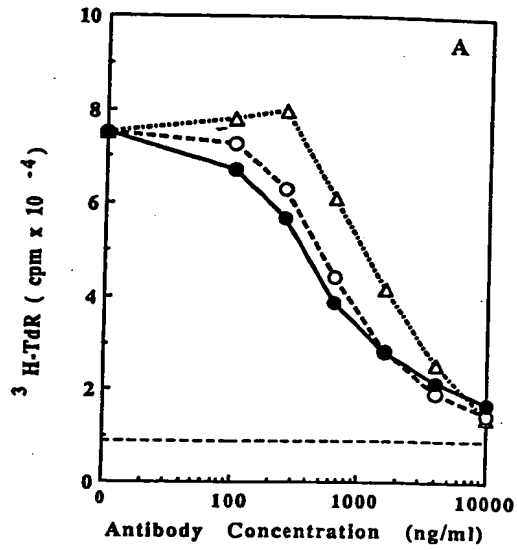


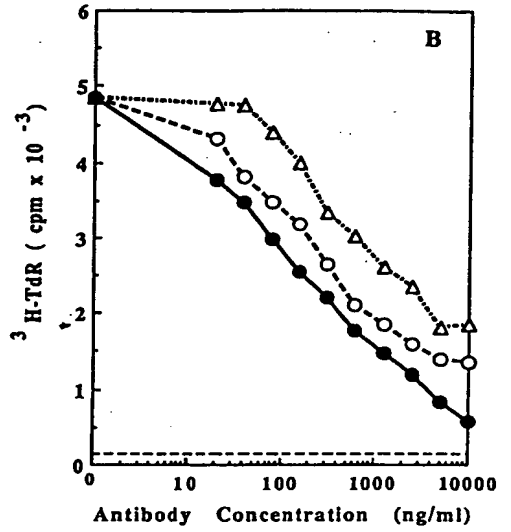
Fig. 4. Analysis of the reshaped human PM-1 antibodies. (*A*) Enzyme-linked immunosorbent assay for binding to human IL-6R. Reshaped antibodies consist of RVla and RVha (Φ), RVhb (□), RVhc (■), RVhd (△), RVhe (▲), and RVhf (●). Chimeric antibody (○) was used as a positive control. (*B*) Enzyme-linked immunosorbent assay testing the ability of antibody to inhibit IL-6 from binding to IL-6R; mouse PM-1 (△), chimeric PM-1 (○), and reshaped human PM-1 consisting of RVla and RVhf (●).

μg/ml (MMS1) in this assay. This was equivalent to the results observed with the original mouse PM-1 antibody, 110 ng/ml, 120 ng/ml, and 1 μg/ml, respectively. These results also indicated that PM-1 antibody is effective on both paracrine and autocrine growth of mutiple myeloma cells through IL-6.

DISCUSSION

We report the design and construction of a reshaped human PM-1 antibody that is equivalent to both mouse and chimeric PM-1 antibodies in terms of antigen binding and growth inhibition of multiple myeloma cells. In the FRs of the best reshaped human PM-1 light chain variable region (version a), there were five differences from the FRs in the original human REI (25) at positions 39, 71, 104, 105, and 107. The three changes in FR4 at positions 104, 105, and 107 were based on a J region from another human κ light chain. The lysine at





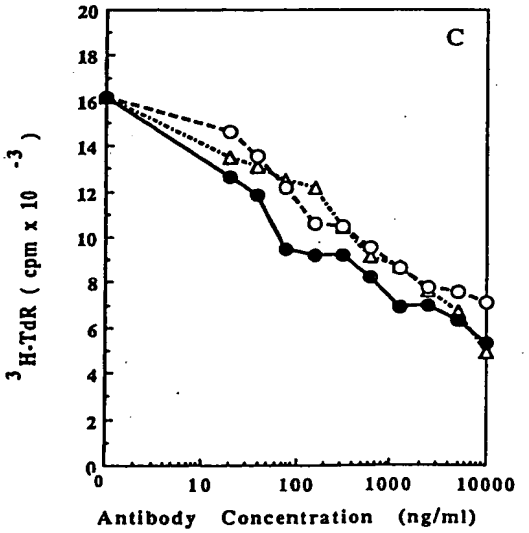


Fig. 5. Comparison of the effect of PM-1 antibodies on multiple myeloma cell growth. (*A*) MMS1 cells; (*B*)ILKM3 cells; and (*C*) S6B45 cells. *Symbols* are the same as in Fig. 4*B*. (– – – –), basal [^{3}H]thymidine uptake of MMS1 and ILKM3 cells in the absence of IL-6.

position 39 is the most common residue from members of the human kappa chain subgroup I. These changes, therefore, do not constitute a deviation from human (7, 34). The tyrosine at position 71 in REI was changed to phenylalanine in the construction of the reshaped human CAMPATH-1H light chain variable region. Since residue 71 is part of the canonical structure for L1 (31), it was predicted that position 71 in the reshaped human PM-1 light chain variable region should be tyrosine as found at that position in mouse PM-1 light chain. Indeed a phenylalanine to tyrosine change at position 71 in the reshaped human D1.3 light chain variable region contributed an additional 0.8 kcal/mol towards stabilization (35). Rather surprisingly, version b of the reshaped human PM-1 light chain variable region (Tyr-71) showed little or n binding to human IL-6R, whereas, version a (Phe-71) showed good binding. In the model of mouse PM-1 variable regions, Tyr-71 is buried and forms a hydrogen bond with Ser-31 on the L1. The loss of the hydrogen bond after changing the tyrosine to phenylalanine seems to have had a beneficial effect on antigen binding, possibly due to conformational changes in L1 which could influence the conformation of the L3 loop.

Of the seven positions tested in the FRs of the reshaped human PM-1 heavy chain variable regions, five changes at positions 27, 28, 29, 30, and 71 were required to recreate a good, functional antigen-binding site comparable to the original mouse PM-1 heavy chain variable region. Residues 27 to 30, although formally designated as part of the FRs (34), are known to form part of the H1 structural loop (31) and possibly interact with the antigen directly. Position 71 is a major determinant of the conformation of the H2 loop. The H2 loop of PM-1 heavy chain variable region consists of a three residue hairpin structure similar to that of HyHEL-10 (33). In the model of the mouse PM-1 variable regions, the Arg-71 interacts with H1 and H2 and could pack against the Ile-29. Indeed Fig. 4 shows that the change of the valine to arginine at position 71 is quite critical in recreating the antigen-binding site. Position 1 was predicted to be a surface residue located close to the CDRs and thus might influence antigen binding. The data comparing versions c and b show that position 1 is probably not very important in recreating the antigen-binding site. The residue at position 48 in the heavy chain variable region was not defined as a canonical residue but the change at this position influenced antigen binding of the reshaped human 425 antibody (9). The data comparing versions d and b show that position 48 is not important in recreating the PM-1 antigen-binding site, possibly due to the difference of the H2 loop structure between PM-1 and 425 antibodies.

As mentioned above, only a few minor changes in FRs were required to reshape a human antibody which is equivalent to the original mouse PM-1 antibody. The reshaped human PM-1 antibody, therefore, looks very like a human antibody and is expected to be a poor immunogen in human patients.

It has been reported that IL-6 is a major myeloma cell growth factor, especially in the final phases of malignant plasma cell dyscrasias (3). Indeed the first clinical study using a mouse monoclonal antibody against human IL-6 to treat a terminally ill patient with primary plasma cell leukemia suggested that inhibitors of IL-6 function could be effective as therapeutic agents for multiple myeloma (4). Mouse PM-1 antibody against human IL-6 receptor has also showed strong antitumor cell activity against multiple myeloma cells *in vivo* (6). The reshaped human PM-1 antibody inhibits multiple myeloma cell growth as well as the original mouse PM-1 antibody does *in vitro* (Fig. 5). *In vivo* efficacy of the reshaped human PM-1 antibody against multiple myeloma will be evaluated. Multiple myeloma is always lethal and very little therapeutic treatment has been achieved. The reshaped human PM-1 antibody is expected to be useful as a therapeutic agent in human multiple myeloma patients.

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